Published in final edited form as:

J Sep Sci. 2009 May ; 32(10): 1605–1612. doi:10.1002/jssc.200900047.

Application of immunoaffinity capillary electrophoresis to the measurements of secreted cytokines by cultured astrocytes

Heather Kalish and Terry M. Phillips

Ultramicro Immunodiagnostics Section, Laboratory of Bioengineering and Physical Science, National Institute of Biomedical Imaging and Bioengineering, National Institutes of Health, Bethesda, MD, USA

Abstract

The ability of the central nervous system (CNS) to act in conjunction with the immune system has been of great interest to both neurobiologists and immunologists. Previous studies have shown that astrocytes can be stimulated, by various peptides, to act as immune regulators within the CNS and release cytokines and chemokines. However, the regulatory mechanism of astrocytes is still poorly understood. Our present study describes a micro-device capable of monitoring the growth and stimulation of 20 astrocytes by vasoactive intestinal peptide. A microdialysis needle was used to collect the secretion by products, which were analyzed by immunoaffinity capillary electrophoresis (ICE) for the secretion of pro-inflammatory cytokines, IL-1 α , IL-1 β , IL-6, and tumor necrosis factor (TNF)- α ; hemopoietic cytokines, IL-3, granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), granulocyte/macrophage colony-stimulating factor (GM-CSF); and chemokines; regulated upon activation normal T-cell expression sequence (RANTES), macrophage inflammatory protein (MIP)-1 α and MIP-1 β . Vasoactive intestinal peptide stimulated astrocytes showed an almost immediate release of pro-inflammatory cytokines and chemokines, with an increase over baseline ranging from 3 to 15 fold, while no substantial increase over baseline was observed for hemopoietic cytokines. This system demonstrates the ability to isolate individual cells in a closely controlled environment and identify and quantify their analytes.

Keywords

Astrocytes; CE; Chemokines; Cytokines; Immunoaffinity

1 Introduction

A neuropeptide is broadly defined as any peptide used by neuronal cells to communicate. Their function, as signaling mechanisms, within the nervous system makes them similar to neurotransmitters, however they appear to have a more significant effect on information processing, modulating neuronal communication by acting on cell surface receptors (www.neuropeptides.nl). Vasoactive intestinal peptide (VIP) is a multifunctional neuropeptide [1] that has a wide range of biological activity, and is quantitatively and functionally the most prominent immunoregulatory neuropeptide that participates in local tissue immune responses [2]. Previous research has shown that VIP can stimulate astrocytes, CD4⁺ lymphocytes and

© 2009 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Correspondence: Dr. Heather Kalish, Ultramicro Immunodiagnostics Section, Laboratory of Bioengineering and Physical Science, National Institute of Biomedical Imaging and Bioengineering, National Institutes of Health, Building 13, Room 3E42, 9000 Rockville Pike, Bethesda, MD, USA kalishH@mail.nih.gov Fax: +1-301-496-6608.

The authors declared no conflict of interest.

microglia, among other cells, to produce cytokines and chemokines during an immunological response [3–8].

It is now accepted that astrocytes have a functional role as immune effector cells within the central nervous system (CNS) [9]. Astrocytes react to neurological insults by producing soluble mediators such as cytokines and chemokines that activate a range of downstream signaling cascades [10]. Cytokines and chemokines are considered to be essential to cellular communication, however their aberrant production by astrocytes contributes to CNS diseases, such as Parkinson's disease and multiple sclerosis [9].

Cytokines and their subclass chemokines, which are specific cytokines that mediate chemoattraction between cells, can be produced by almost all nucleated cells. They are simple peptides or glycoproteins whose sustained and elevated production in the body is often associated with pathologies [10]. Cytokines, such as IL-1 α , IL-1 β , IL-6, tumor necrosis factor (TNF)- α have been associated with a pro-inflammatory response [11,12], cytokines such as IL-3, granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), and granulocyte/macrophage colony-stimulating factor (GM-CSF) are hemopoietic growth factors [13–15] and chemokines such as regulated upon activation, normal T-cell expressed, and secreted (RANTES), macrophage inflammatory protein (MIP)-1 α and MIP-1 β play a vital role in defining the cellular composition of inflammatory infiltrates at the sites of tissue damage [16].

Characterization of the cytokines and chemokines released from cultures has been accomplished by several different techniques including commercial cytometric bead array kits [17], cytokine specific ELISA [16,18], multiplex technology (using a bead-based sandwich immunoassay technique) [19], double color cytokine ELISPOT assays [20], intracytoplasmic cytokine staining [21], electrochemiluminescence method [22], and ICE [8], with ELISA being the most common method used.

CE is a fairly new analytical technique capable of high resolution using low sample and reagent volumes, with results generated in a relatively short amount of time and whose rugged nature can allow direct injection of biofluids with possible reductions in sample pretreatments [23]. Derivatizing the capillary with antibodies to allow for the selective capture and analysis of specific analytes makes ICE a very practical analytical technique for cell secretion analysis. ICE is an alternative to the traditional ELISA assay, offering greater resolving power, using less reagent and sample and yielding lower false positive results [24]. In previous studies, Brenneman *et al.* and Phillips used ICE to isolate and identify a variety of cytokines and chemokines in the secretions of stimulated lymphocytes and astroglia [4–8,25].

In this paper, we present a micro-chamber capable of maintaining a culture of 10 – 20 astrocytes for 2 – 3 days. The micro-chamber allowed for the stimulation of the astrocytes by VIP and the removal of the cell secretions at specific time points following the stimulation. Using ICE, we were able to analyze the cell secretions and identify and quantitate the amount of the following pro-inflammatory cytokines, IL-1 α , IL-1 β , IL-6, and TNF- α ; hemopoietic cytokines, IL-3, G-CSF, M-CSF, GM-CSF; and chemokines; RANTES, MIP-1 α , and MIP1 β being secreted from the stimulated astrocytes.

2 Materials and methods

2.1 Reagents

VIP was obtained from Bachem Biosciences (King of Prussia, PA, USA). Recombinant mouse cytokines (IL-1 α , IL-1 β , IL-6, TNF- α , IL-3, G-CSF, M-CSF, GM-CSF, and human IL-11) and chemokines (RANTES, MIP-1 α , MIP1 β) and their corresponding antibodies were obtained

from R & D Systems (Minneapolis, MN, USA). AlexaFluor 633 was obtained from Molecular Probes, Invitrogen (Carlsbad, California, USA). DMEM, fetal bovine serum (FBS), Trypsin, and Trypan Blue were purchased from ATCC (Manassas, Virginia, USA). The ImmunoPure F(Ab)₂ preparation kit, DTT, and sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SSMCC) were all purchased from Pierce (Rockford, IL, USA). 3-Amino-propyltriethoxysilane was purchased from Polysciences (Warrington, PA, USA). All other chemicals were purchased from Sigma – Aldrich Chemicals (St. Louis, MO, USA). Prior to use, all solutions were passed through a 0.2 µm NC filter (Millipore, Bedford, MA, USA). Standard solutions were prepared as described below and used throughout the experiments. A final concentration of VIP of 1 µg/mL was obtained by dissolving VIP in a 0.01 M phosphate buffer, pH 7.4. The cytokines/chemokines were reconstituted to a final concentration of 1 µg/mL by dissolving the stock solutions in 0.01 M phosphate buffer, pH 7.4. The final concentration of Alexa Fluor 633, of 5 ng/mL, was obtained by dissolving the solid in formamide with sodium bicarbonate to maintain pH 9.0.

2.2 Preparation of cell cultures

Astrocytes, cell line CRL-2541 were purchased from ATCC (Manassas, Virginia, USA). The frozen cells were suspended in DMEM, supplemented with 5% v/v FBS, centrifuged at 800×g for 10 min to remove any freezing media. Cells were suspended in 10 mL DMEM supplemented with 5% FBS media and cultured at 37°C in an atmosphere of 95% cleaned air/5% CO₂, until reaching 2×10⁶ cells. The DMEM/5% FBS media were decanted and the cells were incubated with 5 mL of Trypsin at 37°C for 10 min to detach the cells. The cells were centrifuged at 800×g for 10 min, resuspended in DMEM and the cell suspension was diluted to 200 cells/50 µL and held at 4°C until use.

2.3 Single cell chamber

The single cell micro-incubation chamber, depicted in Fig. 1, was constructed as described previously [8] with a few modifications. A square micro-incubation chamber 20×20 mm², 3 mm thick was cut from plexiglass. A 1.3 mm deep, 1.0 mm diameter hole was drilled into the center of the square chamber to accommodate the cells. Drilled at a right angle to the central well was a 0.5 mm diameter hole, which penetrated the central well at the base and would accommodate a microdialysis probe. A 10 mm long, 0.5 mm OD PES membrane CMA/20 microdialysis probe, 100 kDa cut-off (CMA/Microdialysis, Acton, MA, USA), was placed in the right angle hole and cemented in place with the semi-permeable membrane in contact with the central well. Twenty cells, in 5 µL of DMEM/5% FBS medium, were placed in the central well and a square, glass coverslip cemented in place. The chamber was maintained at 37°C, in an atmosphere of 95% cleaned air/5% CO₂ by placing it in a Narishige open Cunnigham-style micro-incubator (Narishige, Greenvale, NY USA), which was placed on the stage of a Nikon inverted microscope. The single cell chamber was maintained in the micro-incubator on the microscope stage for the duration of the experiment.

2.4 Sample recovery via microdialysis

The samples were recovered from the single cell micro-incubation chamber by microdialysis as previously described [8]. The setup consisted of a Model CMA/102 microdialysis pump, equipped with a single syringe and a CMA/142 fraction collector, both manufactured by CMA/Microdialysis (Acton). The inlet of the microdialysis probe was connected to the syringe by an infusion line, while the outlet of the probe was connected by another line to the fraction collector. Within the chamber, fluid exchange took place between the chamber serum and infused 0.1 mol/L phosphate buffer, pH 7.4, with human IL-11 added as a recovery marker, at a rate of 1 µL/min. The outlet dialysis fluid was collected continuously and every 5 min, or 20

μL, fractions were analyzed. The cells were continuously stimulated with a 10 mmol/L dose of VIP.

2.5 Immunoaffinity capillary preparation

The immunoaffinity capillary was prepared as described previously and graphically depicted in Fig. 2 [26,27]. Briefly, each anticytokine and antichemokine antibody was enzymatically digested into divalent F(Ab)₂ fragments using Pierce Immunopure F(Ab)₂ preparation kits, according to the manufacturer's instructions. Two monovalent FAb fragments resulted from further reduction of the F(Ab)₂ fragments by incubating the F(Ab)₂ with an equal volume of 200 mmol/L DTT for 30 min at 37°C [28]. Each anticytokine and antichemokine FAb were used in equal amounts in a mixture used to coat the inside wall of a single capillary. Capillary preparation began by cutting a fused-silica capillary with an unmodified internal surface and a 100 μm id (Bio-Rad Laboratories, Hercules, CA, USA) to a length of 100 cm and creating a 2 cm detector cell in the capillary, 65 cm to the detector cell, by burning away a section of the external coating. An aqueous solution of 500 nL of 10% 3-aminopropyl-triethoxysilane was introduced by capillary action (the liquid was allowed to diffuse slowly) into the end furthest from the detector cell, considered the inlet, and incubated at 100°C for 60 min. This process was repeated four times, then 10 mmol/L HCl was introduced into the capillary by capillary action and incubated for another 60 min at 100°C. Before continuing with the preparation of the thiol-activated surface, the capillary was washed once with distilled water. SSMCC solution of 500 nL of a 1 mg/mL dissolved in 50 mmol/L sodium borate, pH 7.6 was taken up by capillary action. Once this was completed, the capillary was incubated at 30°C for 60 min, flushed with 50 mmol/L borate, filled by capillary action with the FAb mixture and the capillary was incubated overnight at 4°C. This procedure resulted in approximately a 50-fold excess of each antibody to the expected concentrations of the analytes of interest in the sample. This amount was found previously to allow for the capture of all of the available analytes, in the injected sample, while avoiding significant nonspecific binding during the short antibody capture time (5 min) [8]. The excess antibody also avoided loss of affinity due to the immobilization of FAb's. Because the amount of analyte in the microdialysis fluid was unknown, although estimated to be trace amounts, the immunoaffinity capillary was prepared with ~50% excess antibody to ensure 100% retention of each analyte. To ensure no cross-reactivity of the antibodies, all of them were tested by dot-blot for cross-reactivity against the entire panel of 11 cytokines and chemokines. Once the antibody coating was completed, 100 mmol/L phosphate buffer, pH 7.4 was used to wash the capillary three times. Finally the capillary was mounted onto a Crystal 660 CE system (Prince Technologies, Amsterdam, Netherlands) equipped with a Picometrics LIF detector. The excitation source for the detector was a red light emitting 633 nm helium – neon laser at 12 mW.

2.6 Analysis of the samples

The samples were analyzed as described previously [8]. Briefly, to determine the protein content of each 20 μL fraction, the fraction was measured by direct spectrophotometry at 260/280 nm. The resulting protein content was adjusted to 1 ng/mL by adding phosphate buffer. The samples were then labeled with AlexaFluor 633 by adding 20 μL of the standard AlexaFluor 633 prepared solution. The mixture was placed into black-capped tubes and mixed for 20 min at room temperature. This resulted in circa one dye molecule per analyte, thus reducing interference during the antibody capture phase. After the incubation period, the samples were analyzed by ICE. Known dilutions of each cytokine and chemokine standard were prepared as described above. The calculation of the concentration of each cytokine and chemokine was done by comparison to a standard curve drawn from known amounts of pure cytokines and chemokines run under identical conditions.

2.7 Immunoaffinity capillary electrophoresis analysis of VIP stimulated samples

To determine the identity of the cytokines and chemokines in the samples from VIP stimulated cells, ~30 nL was injected by vacuum injection. This amount was calculated to (i) capture all of the available analytes of interest and (ii) not to overload the detection system. The samples remained in contact with the immobilized FAb's for 5 min, and then the capillary was purged with 200 μ L of 100 mmol/L phosphate buffer, pH 7.4, with 0.01% Brij 35 added. This removed the majority of the unbound material prior to elution of the bound analytes. To elute the bound analytes, the running buffer was changed to 100 mmol/L phosphate buffer, pH 1.5, containing 0.01% Brij 35 and the released analytes separated by applying a 100 μ A constant current. The separated analytes were monitored continuously online by the LIF detector.

3 Results

3.1 Evaluation of the micro-dialysis sampling unit

The micro-dialysis sampling unit was evaluated for efficiency of recovery and reproducibility between runs. With the cell chamber void of cells, the recombinant cytokines and recombinant chemokines were introduced into the inlet of the microdialysis sampling unit. The dialysis inlet, outlet, and semi-permeable membrane showed no signs of clogging or blockage throughout the experiments. The outlet tubing was sampled every 5 min and the amount of chemokines/cytokines recovered was calculated. With an initial sample introduction of 10 pg/mL, recovery rates varied from 89 to 97%. With 100 pg/mL sample introduced, recovery rates varied from 90 to 98%. The recovery rate for each individual analyte was calculated and is presented in Table 1. To ensure the efficiency of the probe was about or above 90% throughout the experiment, each single cell chamber and micro-dialysis probe were used only once. The cell culture chamber was capable of maintaining 20 viable astrocytes for 40 min. VIP was introduced through the inlet of the microdialysis sampling unit and samples containing neuropeptide-induced cytokines/chemokines were recovered from the outlet of the microdialysis sampling unit. Samples were collected every 5 min, from 10 min post-stimulation until 40 min post-stimulation.

3.2 ICE analysis

Approximately 150 ng of the immobilized FAb mixture was determined to be in the ICE capillary, which was stable for 100 – 150 analyses when operated at room temperature. To extend the life of the capillary to ~200 runs, it could be stored at 4°C when not in use and kept between 5 and 10°C throughout the analyses. The ICE system was found to have an LOD of circa 0.1 fg/mL as determined by dilution experiments (results not shown) and a saturation limit of circa 3.4 ng/mL. A typical ICE electropherogram, collected from analysis of the cytokine/chemokine standards, is seen in Fig. 3. The peaks got eluted in the order of their affinity for their corresponding antibody and separated during the electrophoresis stage according to their charge. The absence of extraneous peaks indicated that the washing step following analyte capture was successful. The presence of additional materials other than the analytes of interest would have appeared as additional peaks on the electropherograms.

To calculate the intra-assay c.v. of the ICE system, a mixture containing 100 fg of each cytokine/chemokine was injected five times and the intra-assay was calculated. To calculate the inter-assay c.v., the same sample was run on five consecutive days and the values calculated. The results are tabulated in Table 2.

3.3 Neuropeptide-stimulated single cell secretions

The 20 astrocytes maintained in the single cell micro-incubation chamber were stimulated by VIP and the cell secretions collected every 5 min, until a maximum of 40 min. Figure 4 depicts

a 0 – 40 min time course of electropherograms collected for the cell secretions collected from the VIP stimulated astrocytes. This was repeated with three other chambers, for a total of four chambers, all containing cells from the same cell culture. A second cell culture was divided into four chambers and the experiment repeated, for a total of eight values for each cytokine/chemokine. The secretions were analyzed for pro-inflammatory and hemopoietic cytokines and chemokines and the calculated values, along with their baseline values, from the first experiment are plotted in Figs. 5 – 7. The highest average values for each cytokine/chemokine from the first and second experiment, their SD and the time point when the highest value was reached are shown in Table 3. Once 40 min was reached the cells began to show signs of cell death and the experiment was terminated.

4 Discussion

The function of astrocytes within the CNS as immune regulators is a somewhat controversial subject. While other CNS cells have a defined role in their immune response within the CNS, the role of astrocytes still remains poorly understood. It is becoming clear that astrocytes are active participants in synaptic functioning and exhibit properties, such as the secretion of classical transmitters, previously thought to be exclusively neuronal [29]. Recently, more studies are indicating that astrocytes can be stimulated by neuropeptides to release cytokines and chemokines, which are chemical messengers that set off a cascade of events within the body [18,30,31]. This study has shown that the stimulation of astrocytes by a constant amount of VIP stimulates astrocytes to significantly increase production of almost all cytokines and all chemokines. However, there is no sharp increase seen in IL-3, G-CSF, or M-CSF, which is in contrast to findings previously reported by Brenneman *et al.* [7]. Brenneman *et al.* showed that astrocytes stimulated by different amounts VIP have a significant effect on the amounts of IL-3, G-CSF, or M-CSF released [7]. However experimental difference included pre-incubation of the cells with VIP before collection of samples in contrast to direct stimulation and measurement, the use of cells directly from an animal *versus* commercially available cell lines, and maintaining the cells in a large culture as opposed to a single cell environment.

In order to truly understand how cells work independently of each other, it is important to isolate them at a single cell level and truly understand how they function independent of co-stimulating factors around them, such as other cell types. The frequent assumption in cell and microbiology remains that every cell in a clonal population is identical [32]. However, this is often not the case and there is a developing interest in examining individual cells under controllable conditions [33–35]. The single cell micro-incubation chamber developed in our lab and used in this study affords the opportunity to study astrocytes in the absence of other neuronal or glial cells, under closely controlled conditions and examine the effects of specific neuropeptides on cytokine/chemokine release. This system is similar in purpose to many of the microarray systems being developed for single cell analysis [36,37]; however, it requires no special surfaces or use of electricity to maintain the cells, which may ultimately affect the cells properties [32,34,35,38,39].

CE is an emerging technology with great potential. The low sample requirements, the low LOD, and the time saved in the joint analysis of multiple analytes are an improvement over the existing technologies for the isolation and identification of analytes, such as ELISA. The addition of immunoaffinity separation has increased the sensitivity of CE and can be used to study the affinity constants of immobilized ligands [40]. The ability to concentrate the analytes present in samples, through antibody capture and controlled release, increases the LOD of CE without time consuming and costly pre-processing of samples [41]. Greater sensitivity can be achieved by prolonging the time the sample reacts with the immunoaffinity phase but in many cases, especially when dealing with biological fluids, such prolonged contact also increases nonspecific adherence. Using ICE to separate and identify the cytokines/chemokines released

from VIP stimulated astrocytes allowed the simultaneous identification of 11 analytes in a short amount of time, with little sample workup prior to injection. Technical issues such as high throughput, the use of selective antibodies, and the time to run a sample are issues that still need to be overcome before the technology will find use in clinical laboratories [41], but ICE is a technique that lends itself to even smaller formats on microchips and ultimately “lab on a chip formats” [42,43].

Combining the concentration and separation abilities of ICE with the ability to isolate, stimulate, and monitor single astrocytes provides a comprehensive system for studying cellular mechanisms at the cellular level. By understanding astrocytes at the cellular level a greater understanding of their role in the CNS and specific neurodegenerative diseases, autoimmune diseases etc. can hopefully be advanced.

Acknowledgments

This research was supported by the Intramural Research Program of the National Institute of Biomedical Imaging and Bioengineering, NIH.

Abbreviations

CNS, central nervous system
 FBS, fetal bovine serum
 G-CSF, granulocyte colony-stimulating factor
 GM-CSF, granulocyte/macrophage colony-stimulating factor
 IL, inter-leukin
 ICE, immunoaffinity capillary electrophoresis
 M-CSF, macrophage colony-stimulating factor
 MIP, macrophage inflammatory protein
 RANTES, regulated upon activation normal T-cell expression sequence
 TNF, tumor necrosis factor
 VIP, vasoactive intestinal peptide

5 References

1. Brenneman DE. Peptides 2007;28:1720–1726. [PubMed: 17513014]
2. Yadav M, Goetzl EJ. Ann. N. Y. Acad. Sci 2008;1144:83–89. [PubMed: 19076367]
3. Bailey SL, Carpentier PA, McMahon EJ, Begolka WS, Miller SD. Crit. Rev. Immunol 2006;26:149–188. [PubMed: 16700651]
4. Brenneman DE, Hauser J, Spong CY, Phillips TM. Ann. N. Y. Acad. Sci 2000;921:109–114. [PubMed: 11193813]
5. Brenneman DE, Hill JM, Gozes I, Phillips TM. Ann. N. Y. Acad. Sci 1996;805:280–287. [PubMed: 8993410]discussion 287 – 289
6. Brenneman DE, Phillips TM, Festoff BW, Gozes I. Ann. N. Y. Acad. Sci 1997;814:167–173. [PubMed: 9160969]
7. Brenneman DE, Phillips TM, Hauser J, Hill JM, Spong CY, Gozes I. Neuropeptides 2003;37:111–119. [PubMed: 12747943]
8. Phillips TM. Luminescence 2001;16:145–152. [PubMed: 11312540]
9. Langlais P, Wang CH, Dong LQ, Carroll CA, Weintraub ST, Liu F. Biochemistry 2005;44:8890–8897. [PubMed: 15952796]
10. Ransohoff, RM.; Benveniste, EN. Cytokines and the CNS. Vol. 2nd ed.. CRC Press; Boca Raton, FL: 2006. p. 361
11. Seruga B, Zhang H, Bernstein LJ, Tannock IF. Nat. Rev. Cancer 2008;8:887–899. [PubMed: 18846100]

12. Zhao B, Schwartz JP. *J. Neurosci. Res* 1998;52:7–16. [PubMed: 9556025]
13. Crowe SM, Lopez A. *J. Leukocyte Biol* 1997;62:41–48. [PubMed: 9225991]
14. De Boer ML, Mordvinov VA, Thomas MA, Sanderson CJ. *Int. J. Biochem. Cell Biol* 1999;31:1221–1236. [PubMed: 10582349]
15. Lanza F, Rigolin GM, Castagnari B, Moretti S, Castoldi G. *Haematologica* 1997;82:239–245. [PubMed: 9175331]
16. Besong G, Battaglia G, D'Onofrio M, Di Marco R, Ngomba RT, Storto M, Castiglione M, Mangano K, Busceti CL, Nicoletti FR, Bacon K, Tusche M, Valenti O, Conn PJ, Bruno V, Nicoletti F. *J. Neurosci* 2002;22:5403–5411. [PubMed: 12097492]
17. Kulka M, Sheen CH, Tancowny BP, Grammer LC, Schleimer RP. *Immunology* 2008;123:398–410. [PubMed: 17922833]
18. Ludwig A, Schulte A, Schnack C, Hundhausen C, Reiss K, Brodway N, Held-Feindt J, Mentlein R. *J. Neurochem* 2005;93:1293–1303. [PubMed: 15934948]
19. Lagrelius M, Jones P, Franck K, Gaines H. *Cytokine* 2006;33:156–165. [PubMed: 16529940]
20. Quast S, Zhang W, Shive C, Kovalovski D, Ott PA, Herzog BA, Boehm BO, Tary-Lehmann M, Karulin AY, Lehmann PV. *Cell Immunol* 2005;237:28–36. [PubMed: 16256965]
21. Helms T, Boehm BO, Asaad RJ, Trezza RP, Lehmann PV, Tary-Lehmann M. *J. Immunol* 2000;164:3723–3732. [PubMed: 10725731]
22. Sennikov SV, Krysov SV, Injelevskaya TV, Silkov AN, Grishina LV, Kozlov VA. *J. Immunol. Methods* 2003;275:81–88. [PubMed: 12667672]
23. Altria KD. *J. Chromatogr., A* 1999;856:443–463. [PubMed: 10526799]
24. Guzman NA, Blanc T, Phillips TM. *Electrophoresis* 2008;29:3259–3278. [PubMed: 18646282]
25. Brenneiman DE, Hauser JM, Spong C, Phillips TM. *Neuropeptides* 2002;36:271–280. [PubMed: 12372701]
26. Phillips TM, Dickens BF. *Electrophoresis* 1998;19:2991–2996. [PubMed: 9870401]
27. Phillips TM, Kennedy LM, De Fabo EC. *J. Chromatogr B Biomed. Sci. Appl* 1997;697:101–109. [PubMed: 9342660]
28. Cleland WW. *Biochemistry* 1964;3:480–482. [PubMed: 14192894]
29. Ramamoorthy P, Whim MD. *J. Neurosci* 2008;28:13815–13827. [PubMed: 19091972]
30. Phulwani NK, Esen N, Syed MM, Kielian T. *J. Immunol* 2008;181:3841–3849. [PubMed: 18768838]
31. Miljkovic D, Momcilovic M, Stojanovic I, Stosic-Grujicic S, Ramic Z, Mostarica-Stojkovic M. *J. Neurosci. Res* 2007;85:3598–3606. [PubMed: 17969033]
32. Kortmann H, Blank LM, Schmid A. *Cytometry A* 2008;75A:130–139.
33. Wu LY, Di Carlo D, Lee LP. *Biomed. Microdevices* 2008;10:197–202. [PubMed: 17965938]
34. Tokimitsu Y, Kishi H, Kondo S, Honda R, Tajiri K, Motoki K, Ozawa T, Kadowaki S, Obata T, Fujiki S, Tateno C, Takaishi H, Chayama K, Yoshizato K, Tamiya E, Sugiyama T, Muraguchi A. *Cytometry A* 2007;71:1003–1010. [PubMed: 17972305]
35. Yamamura S, Kishi H, Tokimitsu Y, Kondo S, Honda R, Rao SR, Omori M, Tamiya E, Muraguchi A. *Anal. Chem* 2005;77:8050–8056. [PubMed: 16351155]
36. Balagadde FK, You L, Hansen CL, Arnold FH, Quake SR. *Science* 2005;309:137–140. [PubMed: 15994559]
37. Cai L, Friedman N, Xie XS. *Nature* 2006;440:358–362. [PubMed: 16541077]
38. Di Carlo D, Wu LY, Lee LP. *Lab. Chip* 2006;6:1445–1449. [PubMed: 17066168]
39. Lee PJ, Hung PJ, Rao VM, Lee LP. *Biotechnol. Bioeng* 2006;94:5–14. [PubMed: 16315325]
40. Ostergaard J, Heegaard NH. *Electrophoresis* 2006;27:2590–2608. [PubMed: 16732622]
41. Amundsen LK, Siren H. *Electrophoresis* 2007;28:99–113. [PubMed: 17149780]
42. Phillips TM. *Electrophoresis* 2004;25:1652–1659. [PubMed: 15188254]
43. Wellner E, Kalish H. *Electrophoresis* 2008;29:3477–3483. [PubMed: 18651671]

Figure 1. Drawing of A) top view and B) side view of the single cell micro-incubation chamber with the microdialysis sampling probe cemented in place.

Figure 2.

Illustration of the preparation of an immunoaffinity capillary. Step 1: The antibody is digested by pepsin digestion, yielding the $F(Ab)_2'$ fragment, which is further reduced by Cleland's reagent to 2 FAb fragments. Step 2: The antibody fragments are loaded by capillary action into the derivatized capillary. Step 3: The AlexaFluor labeled analytes are introduced into the capillary and bind to the immobilized antibody. Nonreactive analytes are removed. Step 4: An elution buffer is introduced into the capillary, breaking the antibody/analyte bond and releasing the analytes, which separate and are detected as an electropherogram.

Figure 3.

An electropherogram of the 11 cytokine/chemokine standards run on the ICE system. The IL-11 marker is not shown in the electropherograms.

Figure 4. Electropherograms of cytokine/chemokine secretions from VIP stimulated astrocytes. While samples were collected every 5 min, electropherograms are depicted every 10 min for clarity. Peaks from left to right are: M-CSF, GM-CSF, G-CSF, IL-6, IL-3, TNF- α , IL-1 α , IL-1 β , MIP-1 α , MIP-1 β , and RANTES. The IL-11 marker is not shown in the electropherograms.

Figure 5. Graph of time (min) *versus* amount of analytes recovered (pg/mL) for pro-inflammatory cytokines recovered from VIP stimulated astrocytes.

Figure 6. Graph of time (min) *versus* amount of analytes recovered (pg/mL) for hemopoietic cytokines recovered from VIP stimulated astrocytes.

Figure 7. Graph of time (min) *versus* amount of analytes recovered (pg/mL) for chemokines recovered from VIP stimulated astrocytes.

Table 1

Efficiency of analyte recovery by microdialysis

Analyte	Amount injected (pg/mL)	Amount recovered (pg/mL)
IL-1 α	10	8.9
	100	94.6
IL-1 β	10	9.7
	100	97.2
IL-3	10	9.2
	100	94.9
IL-6	10	9.6
	100	97.8
TNF- α	10	9.8
	100	98.1
G-CSF	10	8.9
	100	93.7
M-CSF	10	9.4
	100	96.1
GM-CSF	10	9.5
	100	96.7
RANTES	10	9.4
	100	98.2
MIP-1 α	10	9.3
	100	92.5
MIP-1 β	10	8.9
	100	90.7

Table 2

The coefficients of variance (CV) for the analytes

Analyte	Intra-assay (%)	Inter-assay (%)
IL-1 α	5.25	5.52
IL-1 β	3.62	4.01
IL-3	6.11	6.53
IL-6	2.89	3.22
TNF- α	3.04	3.38
G-CSF	4.07	4.56
M-CSF	3.76	4.08
GM-CSF	4.26	5.02
RANTES	3.88	4.46
MIP-1 α	2.97	3.17
MIP-1 β	3.75	4.15

Table 3

The highest average concentrations of the cytokine/chemokines in the VIP stimulated astrocyte secretions and the time of the highest concentration

Analyte	Experiment 1 (pg/mL)	Time (min)	Experiment 2 (pg/mL)	Time (min)
IL-1 α	39.2 $\pm$ 7.1	10	24.7 $\pm$ 3.1	10
IL-1 β	46.7 $\pm$ 10.5	10	60.7 $\pm$ 10.9	10
IL-3	14.4 $\pm$ 4.9	10	13.9 $\pm$ 3.2	10
IL-6	13.7 $\pm$ 3.8	15	16 $\pm$ 2.2	10
TNF- α	78.4 $\pm$ 9.2	10	67.8 $\pm$ 6.6	10
G-CSF	11.5 $\pm$ 2.6	20	12.4 $\pm$ 3.2	20
M-CSF	15.9 $\pm$ 5.8	10	24.0 $\pm$ 5.9	15
GM-CSF	12.3 $\pm$ 4.9	15	23.9 $\pm$ 3.5	25
RANTES	75.3 $\pm$ 9.5	20	71.7 $\pm$ 10.7	20
MIP-1 α	135.8 $\pm$ 20.7	10	142.3 $\pm$ 13.6	15
MIP-1 β	79.2 $\pm$ 12.8	15	96.9 $\pm$ 10.0	15